

Research Note—

Insertion of Reticuloendotheliosis Virus Long Terminal Repeat into the Genome of CVI988 Strain of Marek's Disease Virus Results in Enhanced Growth and Protection

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SUMMARY. Marek's disease (MD) is a lymphoproliferative disease of chickens caused by serotype 1 MD virus (MDV). Vaccination of commercial poultry has drastically reduced losses from MD, and the poultry industry cannot be sustained without the use of vaccines. Retrovirus insertion into herpesvirus genomes is an efficient process that alters the biological properties of herpesviruses. RM1, a virus derived from the virulent JM strain of MDV, by insertion of the reticuloendotheliosis (REV) long terminal repeat (LTR), was attenuated for oncogenicity but retains properties of the parental virus, such as lymphoid organ atrophy. Here we show that insertion of the REV LTR into the genome of vaccine strain CVI988 resulted in a virus (CVRM) that replicated to higher levels than parental CVI988 in cell culture and that remained apathogenic for chickens. In addition, CVRM showed protection indices similar or superior to those afforded by CVI988 virus in laboratory and field protection trials, indicating that it could be developed as a safe and efficacious vaccine to protect against very virulent plus MDV.

RESUMEN. *Nota de Investigación*—La inserción de repeticiones terminales largas del virus de la reticuloendoteliosis en el genoma de la cepa CVI988 del virus de Marek resultó en un aumento en el crecimiento y en la protección.

La enfermedad de Marek (MD) es una enfermedad linfoproliferativa de los pollos causada por el serotipo 1 del virus de Marek (MDV). La vacunación de las aves de corral comerciales ha reducido drásticamente las pérdidas por el virus de Marek, y la industria avícola no puede operar sin el uso de vacunas. La inserción de retrovirus en los genomas de los virus del herpes es un proceso eficiente que altera las propiedades biológicas de los herpesvirus. El virus RM1, que es un virus derivado de la cepa virulenta JM mediante la inserción de repeticiones terminales largas del virus de la reticuloendoteliosis (REV) fue atenuado en su oncogenicidad, pero conserva propiedades del virus progenitor, tales como atrofia de los órganos linfoides. En este trabajo se muestra que la inserción de la repetición terminal larga del virus de la reticuloendoteliosis en el genoma de la cepa vacunal CVI988 resultó en un virus (CVRM) que replica en cultivo celular a niveles más altos que los virus progenitores CVI988 y se mantuvo apatógeno para los pollos. Además, el virus CVRM mostró índices de protección similares o superiores a los obtenidos por los virus CVI988 en los ensayos de protección de laboratorio y de campo, lo que indica que podría ser desarrollado como una vacuna segura y eficaz para proteger contra las cepas muy virulentas plus del virus de Marek.

Key words: Marek's disease virus, CVI988, vaccine, pathogenesis, protection, LTR

Abbreviations: CVRM = CVI988 virus with an LTR insertion; DEF = duck embryo fibroblasts; IRS = internal repeat short region; LTR = long terminal repeat; MD = Marek's disease; MDV = Marek's disease virus; PFU = plaque-forming units; PI = protective index; REV = reticuloendotheliosis virus; rMd5 = recombinant Md5 virus; TRS = terminal repeat short region; US = unique short region; vv = very virulent; vv+ = very virulent plus

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by serotype 1 MD virus (MDV-1), an alphaherpesvirus. Vaccination of commercial poultry has drastically reduced losses from MD, and the poultry industry cannot be sustained without the use of vaccines. Interestingly, reliance on these vaccines is believed to have contributed to MDV-1 evolution toward greater virulence. However, to control highly virulent field strains there is a need to develop improved vaccines.

With advances in bacterial artificial chromosome and cosmid technologies it is now possible to manipulate the MDV genome with relative ease, generating recombinants with reduced virulence and/or increased protection. For example, a DNA vaccine containing the genome from a cell culture attenuated strain of MDV-1 was able to protect chickens against challenge (12). We have earlier shown that

deletion of the vIL8 gene from recombinant Md5 virus (rMd5), a very virulent (vv) strain, is attenuated in maternal antibody-positive chickens and is able to protect against challenge with a highly virulent MDV strain (1). However, the vIL8 deletion mutant still had residual virulence and could not be used as a commercial vaccine. On the other hand, deletion of the meq gene from rMd5 fully attenuated the virus (8), and the resulting virus, rMd5ΔMeq, provided protection superior to CVI988 (the most efficacious MD vaccine presently available) following challenge with highly virulent MDV strains under both laboratory and field conditions (6,7). These examples show that gene deletion holds promise in terms of developing improved MD vaccines.

Another strategy with which to alter the biological properties of viruses in order to develop improved vaccines is by insertion of sequences that have promoter or enhance functions, thereby altering MDV gene expression. Retrovirus insertion into herpesvirus genomes is an efficient process that alters the biological properties of these viruses. RM1, a virus derived from the virulent strain JM by insertion of the reticuloendotheliosis virus (REV) long terminal repeat (LTR)

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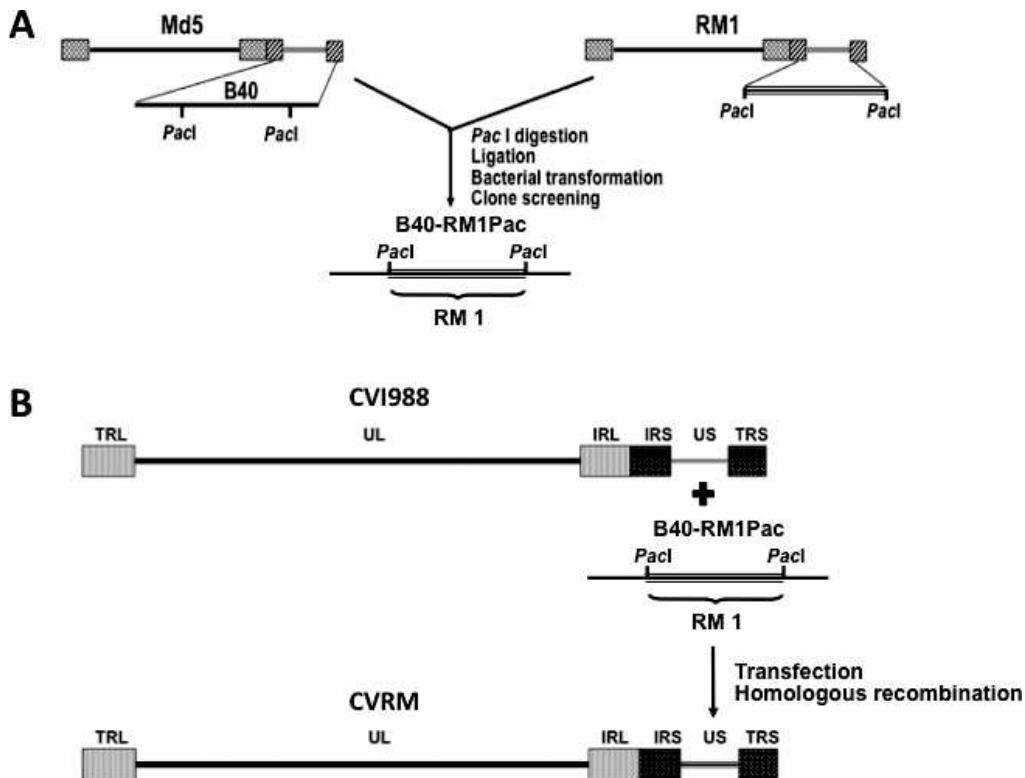


Fig. 1. Generation of CVRM virus. (A) Generation of B40-RM1Pac cosmid containing the *Pacl* segment with LTR of RM1 virus. (B) Generation of CVRM by homologous recombination of CVI988 and B40-RM1Pac cosmid.

around the junction of the unique short region (US) and internal repeat short (IRS) and terminal repeat short (TRS) regions of the genome (4), is attenuated for oncogenicity but retains other *in vivo* properties of virulent viruses, such as lymphoid organ atrophy, efficient early cytopathic infection, and contact spread (13). Similarly, rMd5ΔMeq is completely attenuated for oncogenicity but causes significant immunosuppression in infected chickens (5). Although chickens vaccinated with RM1 or rMd5ΔMeq were protected against challenge with very virulent plus (vv+) MDV, and although levels of protection exceeded those of CVI988, both of these viruses retained the immunosuppressive traits of their parental viruses, making them unsuitable for commercial use.

Since CVI988 strain does not cause immunosuppression in vaccinated chickens, we generated a recombinant CVI988 virus with the LTR insertion from RM1 (CVRM) to further improve vaccine efficacy. Our results indicate that CVRM has increased *in vitro* replication when compared to CVI988, does not induce lymphoid organ atrophy, and shows protection indices equal or superior to those of CVI988, indicating that it can be developed as an effective vaccine to control vv+ field strains.

MATERIALS AND METHODS

Generation of a CVI988 virus with an LTR insertion (CVRM). Genomic DNA isolated from purified RM1 virus was digested with *Pacl*, and restriction fragments were separated on a 0.8% agarose gel. A 15,500-bp fragment containing the US region, part of the IRS and TRS regions, and the LTR was gel purified and ligated to *Pacl*-digested B40 cosmid DNA from the vv strain Md5 (10) to generate cosmid B40-RM1Pac (Fig. 1A). Recombinant CVI988 virus containing the REV LTR (CVRM) was generated by homologous recombination of *NotI* linearized B40-RM1Pac cosmid and genomic DNA isolated from CVI988 infected chicken embryo fibroblasts (Fig. 1B).

Growth characteristics of CVRM in cell culture. *In vitro* growth characteristics of CVRM were evaluated as previously described (10). Briefly, approximately 100 plaque-forming units (PFU) of the different viruses were inoculated onto duck embryo fibroblast (DEF) cells seeded onto 60-mm plates. On days 1, 2, 3, 4, and 5 postinoculation the infected cells were trypsinized, serial dilutions were inoculated onto fresh DEF cells seeded on 35-mm plates, and plaques in the different dilutions were counted 7 days later.

Chickens. *In vivo* characterization of CVRM virus was carried out in F₁ progeny (15×7) chickens of line 15I₅ males and line 7₁ females. For some experiments, these were from breeder hens free of maternal antibody (Ab-), while for others the breeder hens were vaccinated with all three MD vaccine serotypes and were considered positive for maternal antibodies (Ab+). For field protection studies Hy-Line Ab+ chickens, variety W-36 were used.

Evaluation of CVRM pathogenesis in MDV Ab- chickens. To study the pathogenic properties of CVRM, *in vivo* experiments were carried out in MDV Ab- chickens. Chickens were inoculated at day of age by the subcutaneous route with 2000 PFU of CVRM, CVI988, RM1, or rMd5 and maintained in modified Horsfall-Bauer isolators for 8 wk with access to food and water. Mortality during the course of the experiment was recorded and chickens were examined for gross MD lesions. All surviving chickens were euthanized at 56 days postchallenge and examined for gross MD lesions.

Effect of CVRM on lymphoid organ weights. Four groups of 1-day-old Ab- 15×7 chickens were inoculated by the subcutaneous route with 2000 PFU of rMd5, RM1, CVRM, or CVI988. One group remained uninoculated and served as a negative control. To determine the relative lymphoid organ weights, 13 days postinfection, five chickens from each group were euthanized, and the body and lymphoid organs (bursa, spleen, and thymus) weights for each chicken were determined.

Vaccine experiments. To study the protection efficacy of CVRM in the laboratory setting, day-old Ab+ 15×7 chicks were vaccinated with 2000 PFU of CVRM or CVI988 vaccine virus by the intra-abdominal route. Five days later, vaccinated and unvaccinated control chickens were

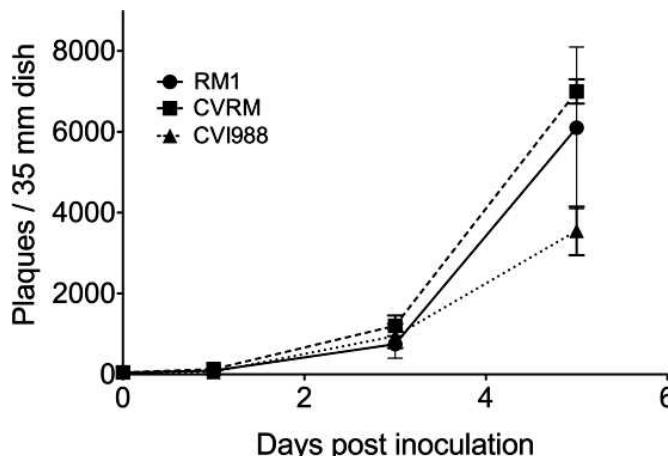


Fig. 2. Growth characteristics of CVRM in cell culture. DEF cells were infected with 100 PFU of either virus, and virus titers were determined at the indicated times postinoculation in fresh DEF cells. Each time point represents the mean of two independent experiments. Bars represent standard error.

challenged by the intra-abdominal route with 500 PFU of vv+ 648A MDV. Mortality during the course of the experiment was recorded, and chickens were examined for gross MD lesions. All surviving chickens were euthanized at 56 days postchallenge and examined for gross MD lesions. Vaccine efficacy was expressed as protective index (PI) and calculated as the percent gross MD in nonvaccinated challenged control chickens minus the percentage of gross MD in vaccinated, challenged chickens divided by the percentage of gross MD in nonvaccinated challenged control chickens $\times 100$.

Effect of vaccination on challenge virus load. To determine the effect of vaccination on challenge virus load, day-old Ab- 15 \times 7 chicks were vaccinated with 2000 PFU of CVRM or CVI988. Five days later, groups of vaccinated and unvaccinated control chickens were challenged by the intra-abdominal route with 500 PFU of vv rMd5. Chickens were bled at 1, 3, and 7 wk postchallenge, and buffy-coat cells were obtained by centrifugation. To determine virus load, 10^6 lymphocytes were added to freshly seeded DEF monolayers and visible viral plaques were counted 5–6 days postinfection.

Field protection studies. A field trial to determine the protection efficacy of CVRM was carried out at a Hy-Line International remote facility (Iowa). Groups of 200 commercial white leghorn Ab+ chickens (variety W-36) were identified by wing bands and vaccinated at hatch by the subcutaneous route with 2000 PFU/chicken of three different CVI988 preparations (A, B, and C), bivalent commercial HVT+SB1 vaccine, or CVRM. Challenge was accomplished by placing vaccinated and nonvaccinated control chicks—approximately 18 hr following vaccination—into a brooder facility that contained 11,000–14,000 three-to-five-wk-old seeder chickens previously inoculated (2 wk prior) with 500 PFU of 686 vv+ MDV at 1 wk of age. Morbidity and

mortality from week 4 through week 18 postvaccination was monitored and recorded. Chickens with typical MD symptoms and/or lesions were recorded as attributable to MD unless there was another obvious primary cause of mortality. The PI was calculated as indicated above.

RESULTS AND DISCUSSION

Retrovirus insertion into herpesvirus genomes is an efficient process that alters the biological properties of herpesviruses. RM1, a virus derived from virulent JM by insertion of the REV LTR close to the junction of the US with the IRS and TRS regions of the genome (4), is attenuated for oncogenicity but retains properties of the parental virus, such as lymphoid organ atrophy, efficient early cytopathic infection, and contact spread (13). Since vaccine virus CVI988 is fully attenuated and highly protective, we sought to investigate if the addition of the LTR of REV into the CVI988 genome (CVRM) would further improve the protection efficacy of CVI988.

CVRM was generated by transfection of linearized cosmid B40-RM1Pac DNA with DNA of CVI988-infected cells resulting in insertion of a single LTR copy in the CVI988 genome by homologous recombination. A single-copy LTR was detected during the first four passages of virus replication, as determined by PCR analysis using primers that span across the LTR region (data not shown). Since the resulting recombinant virus (CVRM) had enhanced growth kinetics compared to parental CVI988, it became the predominant virus without the need for plaque purification. After eight passages in chicken embryo fibroblasts, the majority (>95%, as measured by PCR amplification) of the virus population had two copies of the LTR. Examination of CVRM growth in cell culture indicated that the presence of LTR in the CVRM genome enhanced the replication of the virus to levels similar to RM1 and levels higher than those associated with the parental CVI988 strain, although the differences were not statistically significant (Fig. 2). We believe this replicative advantage may aid in vaccine manufacturing, reducing cost and manufacturing time.

Pathogenicity studies of CVRM in Ab- (data not shown) and Ab+ 15 \times 7 chickens (Table 1) indicated that insertion of the LTR in the CVI988 genome did not increase its pathogenicity. These results are in agreement with those previously reported for JM (13), Md5 (9), and Chinese field isolates (11), in which insertion of the REV LTR in the viral genome resulted in decreased pathogenicity.

Earlier studies (5,6,13) have shown that RM1 and rMd5Δmeq are the most efficacious experimental vaccines, providing superior protection against vv+ MDV challenge. However, both these viruses were found unsafe for commercial use because they cause severe bursal and thymic atrophy. To determine if the insertion of an REV LTR in the genome of CVI988 results in increased lymphoid organ

Table 1. Protection studies of recombinant CVRM vaccine virus compared to commercial CVI988 vaccine strain in 15 \times 7 MDV maternal antibody-positive (Ab+) chickens.^A

Vaccine/challenge	Experiment 1		Experiment 2		Average MD (%) ^B	Average PI
	MD (%)	PI	MD (%)	PI		
No vaccine control	0/17 (0)	NA	0/13 (0)	NA	0	NA
CVRM	0/10 (0)	NA	0/9 (0)	NA	0	NA
CVI988	0/10 (0)	NA	ND	NA	0	NA
CVRM/648A	1/17 (6)	93	4/17 (24)	85a	15ab	85
CVI988/648A	7/17 (41)	53	6/17 (35)	63a	38ab	63
None/648A	15/17 (88)	NA	17/17 (100)	NA	94b	NA

^AMD = number of chickens that developed Marek's disease (%); PI = protective index; NA = not applicable.

^B% MD among the three experimental groups with different lowercase letters differ significantly based upon Fisher exact test ($P < 0.05$).

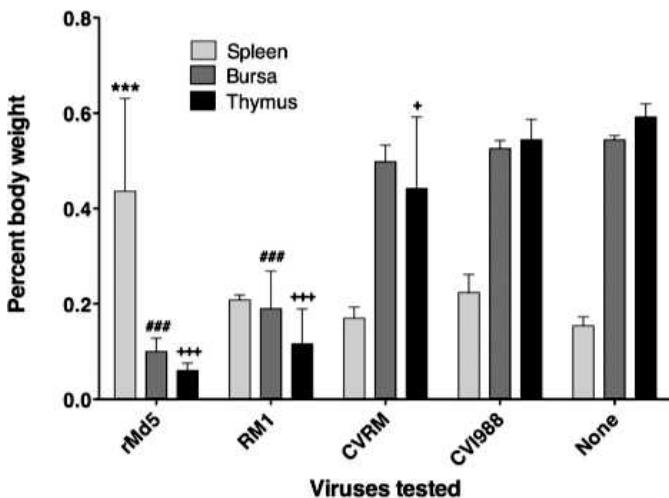


Fig. 3. Relative weight of spleen, bursa of Fabricius, and thymus. One-day-old maternal antibody-negative (15×7) chickens were infected with 2000 PFU of rMd5, RM1, CVRM, or CVI988. Columns represent mean lymphoid organ weights (calculated as a percentage of whole body weight) from five chickens per experimental group. Percent body weight for each organ and vaccine groups were compared by one-way ANOVA followed by Tukey honestly significantly different test. Bars represent 95% confidence intervals (CIs). Columns with a symbol are statistically significant ($P \leq 0.05$) from uninoculated controls. Control group data (rMd5, none) previously reported (5).

atrophy, Ab- 15×7 chickens were inoculated with 2000 PFU of rMd5, RM1, CVRM, or CVI988. As shown in Fig. 3, the relative bursa and thymus weights of CVRM-inoculated chickens were comparable to those of CVI988-inoculated or uninoculated control chickens, while, as expected, significant atrophy was observed in rMd5- and RM1-inoculated chickens. These results show that compared to RM1 (13) and naturally occurring Chinese isolates (2,11), insertion of LTR sequences in the MDV genome did not have a detrimental effect.

To determine if the insertion of REV LTR had an effect on the protective efficacy of CVI988, two protection studies were carried out in Ab- 15×7 chickens. Groups of day-old chicks were vaccinated with CVRM or CVI988, and 5 days postvaccination, chickens were challenged with 500 PFU of vv+ 648A. The average MD incidence for CVRM-vaccinated chickens challenged with 648A was 15%, compared to 38% for the CVI988-vaccinated group, but these results were not statistically significant ($P < 0.05$). As expected, no MD was observed in CVI988- or CVRM-infected chickens, and challenge virus resulted in 100% MD in the nonvaccinated control group (Table 1). These data show that under laboratory settings CVRM confers similar protection against challenge with vv+ 648A virus when compared to CVI988.

Table 3. Field protection studies of recombinant CVRM vaccine virus compared to commercial bivalent and serotype 1 CVI988 vaccine strains in commercial maternal antibody-positive chickens.^A

Group	No.	MD (%) ^B	PI
Unvaccinated control ^C	195	157 (81)a	NA
HVT/SB-1 ^C	197	69 (35)b	56
CVI988 (A) ^C	188	20 (11)c	86
CVI988 (B) ^C	197	72 (37)b	55
CVI988 (C) ^C	194	36 (19)d	77
CVRM	195	23 (12)cd	85

^AMD = number of chickens that developed Marek's disease (%); PI = protective index; NA = not applicable.

^B% MD among the experimental groups with different lowercase letters differ significantly based upon Fisher exact test ($P < 0.05$).

^CControl group data previously reported (6).

It has been shown (3) that the load of challenge MDV DNA in blood can be used as an early criterion in the diagnosis of MD in vaccinated chickens. To examine if vaccination with CVRM caused reduction of challenge virus load, Ab- day-old chicks were vaccinated with CVRM or CVI988 or remained unvaccinated. At 5 days postvaccination, chickens were challenged with 500 PFU of vv rMd5 virus. At 1, 3, and 7 wk postchallenge, five chickens from each group were bled, and cell-associated MDV viremia in peripheral mononuclear blood lymphocytes was determined. As shown in Table 2, both viruses caused a significant and sustained reduction in the level of challenge virus viremia up to 7 wk postchallenge. At 1 wk after challenge with rMd5, the unvaccinated group had a viremia titer of $330 \text{ PFU}/1 \times 10^6$ peripheral mononuclear blood lymphocytes, whereas the CVRM- and CVI988-vaccinated groups had 1 (99.7% reduction) and 21 (93.6% reduction) PFU, respectively, values that were significantly different ($P < 0.05$) from those of the nonvaccinated group. At 3 wk postchallenge, the reduction of challenge virus was 98.7% and 79.9% for CVRM and CVI988 vaccines, respectively, which were significantly different from each other and the nonvaccinated control group, while at 7 wk, the reduction was 98.1% and 98.4%, respectively. These results show that significant reduction of challenge virus replication correlates well within vaccinal protection.

To determine the protection efficacy of CVRM under field conditions, protection conferred by CVRM was compared with that of three different CVI988 preparations and a commercial bivalent HVT+SB-1 vaccine. As shown in Table 3, 80.5% of the unvaccinated chickens died of MD as a result of contact infection with 686 vv+ MDV. In the same brooder facility, 35% of the chickens vaccinated with bivalent vaccine HVT+SB-1 died of MD (PI value of 56), while 10.6%, 18.6%, and 36.5% of the chickens vaccinated with three different sources of CVI988 (A, C, and B, respectively) died of MD (PI values of 86, 77, and 55, respectively).

Table 2. Reduction of viremia in MDV maternal antibody-negative chickens vaccinated and challenged with vv rMd5 virus.^A

Vaccine	Weeks postchallenge					
	1		3		7	
	PFU	% Reduction	PFU	% Reduction	PFU	% Reduction
None ^B	330	NA	149	NA	258	NA
CVRM	1	99.7	2	98.7	5	98.1
CVI988 ^B	21	93.6	30	79.9	4	98.4
None/no challenge	0	—	0	—	0	—

^AChicks were vaccinated at day of age with 2000 PFU of the indicated vaccines and challenged 5 days later with 500 PFU of vv rMd5 virus. NA = not applicable.

^BControl group data previously reported (7).

On the other hand, 12% of chickens vaccinated with CVRM died of MD (PI value of 85), which is comparable to the most efficacious CVI988 (source A) and significantly different from CVI988 (B and C) and the bivalent HVT+SB-1 vaccines tested ($P < 0.05$).

In summary, our results show that CVRM, a CVI988-derived virus with REV LTR insertions in the same location as RM1, replicated with faster kinetics than parental virus *in vitro* and remained apathogenic *in vivo*. In addition, CVRM showed protection indices similar or superior to those afforded by CVI988 virus, indicating that it could be developed as a safe and efficacious vaccine with which to protect against vv+ MDV.

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